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(71) Applicant (for all designated States except US): APHTON
CORPORATION [US/US]; 8 Penn Center, Suite 2300,
1628 JFK Boulevard, Philadelphia, PA 19103 (US).

(72) Inventor; and

(75) Inventor/Applicant (for US only): GRIMES, Stephen
[US/US]; 551 Rutgers Drive, Davis, CA 95616 (US).

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(54) Title: MONOCLONAL ANTIBOLIES TO PROGASTRIN

(57) **Abstract:** The present invention provides progastrin-binding molecules specific for progastrin that do not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly). Further, the invention provides monoclonal antibodies (MAbs) selective for sequences at the N-terminus and the C-terminus of the gastrin precursor molecule, progastrin and the hybridomas that produce these MAbs. Also provided are panels of MAbs useful for the detection and quantitation of progastrin and gastrin hormone species in immuno- detection and quantitation assays. These assays are useful for diagnosing and monitoring a gastrin-promoted disease or condition, or for monitoring the progress of a course of therapy. The invention further provides solid phase assays including immunohistochemical (IHC) and immunofluorescence (IF) assays suitable for detection and visualization of gastrin species in solid samples, such as biopsy samples or tissue slices. The progastrin-binding molecules are useful therapeutically for passive immunization against progastrin in progastrin-promoted diseases or conditions. Also provided are surrogate reference standard (SRS) molecules that are peptide chains of from about 10 to about 35 amino acids, wherein the SRS molecule comprises at least two epitopes found in a protein of interest of greater than about 50 amino acids. Such SRS molecules are useful as standards in place of authentic proteins of interest.

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MONOCLONAL ANTIBODIES TO PROGASTRIN

FIELD OF THE INVENTION

The invention relates to antibodies directed against specific regions of the gastrin hormone precursor, progastrin, found *in vivo* in an animal, particularly a human. The invention further relates to the application of these monoclonal antibodies (MAbs) to detection, diagnosis and monitoring of gastrin-promoted diseases and conditions, and to methods of use of the MAbs of the invention for the prevention and treatment of gastrin-promoted diseases and conditions. The invention also relates to surrogate molecules and their use as reference standards in immunoassays, particularly as applied to peptide hormones.

BACKGROUND OF THE INVENTION

Human preprogastin, a peptide of 101 amino acids, is the primary translation product of the gastrin gene and has the following structure:

MQRLCVYVLI FALALAAFSE ASWKPRSQQP DAPLGTGANR DLELPWLEQQ
GPASHHRRQL GPQGPPHLVA DPSKKQGPWL EEEEEAYGWM DFGRRSAEDE N (SEQ ID NO: 1).

Progastrin is formed by cleavage of the first 21 amino acids (constituting the signal peptide) from preprogastin. The 80 amino acid long chain of progastrin is further processed by cleavage and modifying enzymes to several biologically active gastrin hormone forms, including gastrin-17 (G17), gastrin-34 (G34), glycine extended gastrin-17 (G17-Gly), and glycine extended gastrin-34 (G34-Gly).

Mature G17 is modified at both amino- and carboxy-terminal residues: the N-terminal glutamic acid is cyclized to form pyroglutamic acid (pGlu) and the free carboxyl group of the C-terminal phenylalanine residue is amidated by the enzyme, peptidyl α -amidating mono-oxygenase (PAM) to form a C-terminal Phe-NH₂. Mature G34 is identically amidated at its C-terminal end to form a C-terminal Phe-NH₂. (See Dockray et al., Ann. Rev. Physiol. (2001) 63: 119-139).

Mature G17, the predominant form of "little" gastrin in humans, has the amino acid sequence: pEGPWLEEEEEAYGWMDF-NH₂ (SEQ ID NO: 2). G17-Gly is an incompletely processed form of gastrin found as a minor component of "little" gastrin in healthy human subjects and has the amino acid sequence: pEGPWLEEEEEAYGWMDFG (SEQ ID NO: 3).

Gastrin-34, the predominant form of "big" gastrin in humans, has the amino acid sequence: pELGPQGPPHLVADPSKKEGPWLEEEEEAYGWMDF-NH₂ (SEQ ID NO: 4). Glycine-extended gastrin 34 (G34-Gly) has a C-terminal glycine residue, and has the amino acid sequence: pELGPQGPPHLVADPSKKEGPWLEEEEEAYGWMDFG (SEQ ID NO: 5).

Gastrin is secreted by the pyloric antral-G cells of the stomach in response to gastrin-releasing peptide (GRP), and is suppressed by gastric acid and the paracrine action of several peptide hormones, most notably, somatostatin. It has long been recognized that gastrin peptides function to

stimulate acid secretion in the stomach of healthy individuals, however, it has only recently been shown that these peptides also control proliferation, differentiation and maturation of different cell types in the gastrointestinal (GI) system.

Progastrin is normally fully processed to gastrin hormone forms. When produced in excess, progastrin is at least partly processed to one or more forms of gastrin hormone that act on the gastrointestinal system and may potentiate the formation of gastrin-promoted tumors. In some cases the progastrin is circulated in the blood and can be detected in the urine of patients suffering from progastrin-promoted diseases or conditions.

In addition to their local activity in the GI system, G17 and, to a lesser extent, G17-Gly are released into the bloodstream and have been found to increase in the serum of patients afflicted with gastrointestinal disorders and diseases, such as gastric cancer, colorectal cancer, and pancreatic cancer. These gastrin species have more recently also been found to be associated with other diseases not associated with the gastrointestinal tract, including small cell lung cancer (SCLC) and liver metastasized tumors. See for example "Gastrin and Colon Cancer: a unifying hypothesis" S. N. Joshi et al., *Digestive Diseases* (1996) 14: 334-344; and "Gastrin and Colorectal Cancer" Smith, A.M. and Watson, S.A. (2000) *Alimentary Pharmacology and Therapeutics* 14(10): 1231-1247.

Antibodies are key reagents in numerous assay techniques used in medical, veterinary and other immunodetection fields. Such tests include many routinely used immunoassay techniques, such as for example, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), immunohistochemistry (IHC), and immunofluorescence (IF) assays.

Anti-gastrin polyclonal antibodies have been shown to be effective in inhibiting gastrin activity ("Inhibition of gastrin activity by incubation with antibodies to the C-terminal tetrapeptide of gastrin" Jaffe et al., *Surgery* (1969) 65(4):633-639); and non-human anti-gastrin polyclonal antibodies have been applied to therapy in a patient suffering from Zollinger-Ellison syndrome, a pathological condition in which excessive gastrin is produced without stimulation by feeding. See Hughes et al., "Therapy with Gastrin Antibody in the Zollinger-Ellison Syndrome" Hughes et al., *Digestive Diseases* (1976) 21(3):201-204. However, these rabbit anti-gastrin antibodies had "at best a short term effect in this patient." (Hughes at p. 204). U.S. patents 5,886,128 and 5,785,970 disclose methods of treatment of ulcers or tumors whose growth is dependent on or stimulated by gastrin hormones by immunizing with gastrin hormone peptide conjugates.

Until now, MAbs capable of sensitively detecting, and accurately distinguishing progastrin from the processed forms of gastrin hormone have not been available. Furthermore, until the present invention, it was not possible to accurately measure the amount progastrin in a sample, such as for instance a sample of biological fluid. The MAbs of the invention can be used in assays for clinical testing to precisely define the biology of progastrin in normal and disease states. The invention also provides MAb compositions for pharmaceutical use and methods for the prevention and treatment of progastrin-promoted diseases and conditions.

SUMMARY OF THE INVENTION

The present invention provides a progastrin-binding molecule that selectively binds progastrin, wherein the molecule does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly). The progastrin-binding molecule may be an antibody molecule, such as a monoclonal antibody, an antibody binding region, or a single chain antibody.

In one aspect, the invention provides a monoclonal antibody (MAbs) that selectively binds progastrin at an epitope within the amino acid sequence 1-9 of progastrin, i.e. SWKPRSQQP (SEQ ID NO: 6).

Hybridomas that produce the MAbs that selectively bind progastrin at an epitope within the amino acid sequence 1-9 of progastrin, i.e. SWKPRSQQP (SEQ ID NO: 6) are also provided.

In another aspect, the present invention provides a MAb that selectively binds progastrin at an epitope within the amino acid sequence 6-14, i.e. SQQPDAPLG (SEQ ID NO: 7). Hybridomas that produce MAbs that selectively bind progastrin at an epitope within the amino acid sequence 6-14, SQQPDAPLG (SEQ ID NO: 7) are also provided.

In yet another aspect, the present invention provides a MAb that selectively binds progastrin at an epitope within the amino acid sequence 72-80 of progastrin, GRRSAEDEN (SEQ ID NO: 8). Hybridomas that produce MAbs that selectively bind progastrin at an epitope within the amino acid sequence 72-80, GRRSAEDEN (SEQ ID NO: 8) are also provided.

According to the present invention, combinations of two or more of the a progastrin, wherein the molecule does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly) can be used in a panel of progastrin-binding molecules.

Also provided are pharmaceutical compositions of a progastrin-binding molecule, wherein the molecule does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly) in combination with a pharmaceutically acceptable carrier. In a particular aspect, the invention provides pharmaceutical compositions of a MAb that selectively binds: (1) progastrin at an epitope within the amino acid sequence corresponding to amino acids 1-9 of progastrin, SWKPRSQQP (SEQ ID NO: 6); (2) progastrin at an epitope within the amino acid sequence 6-14, SQQPDAPLG (SEQ ID NO: 7); or (3) progastrin at an epitope within the amino acid sequence of 72-80, GRRSAEDEN (SEQ ID NO: 8); in combination with a pharmaceutically acceptable carrier.

The present invention still further provides a progastrin immunoassay. The method includes: first, obtaining a sample to be assayed for progastrin and contacting the sample with a progastrin-binding molecule that does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly), under suitable conditions for binding and allowing any progastrin present to form a progastrin-progastrin-binding molecule complex; then detecting the presence or absence of the progastrin-progastrin-binding molecule complex; and/or determining the amount of progastrin-progastrin-binding molecule complex in the sample by an immunoassay method.

The present invention further provides methods of diagnosing a gastrin-promoted disease or condition in a patient by determining the level of progastrin in a sample of a biological fluid from the patient and comparing the level of progastrin in the sample with the level of progastrin in a sample of biological fluid from one or more control individuals or with a reference standard. Such gastrin-promoted diseases or conditions can be prevented or treated by administering to a patient in need thereof a pharmaceutical composition including a progastrin-binding molecule that selectively binds: (1) progastrin at an epitope within the amino acid sequence corresponding to amino acids 1-9 of progastrin, SWKPRSQQP (SEQ ID NO: 6); (2) progastrin at an epitope within the amino acid sequence 6-14 of progastrin, SQQPDA PLG (SEQ ID NO: 7); or (3) progastrin at an epitope within the amino acid sequence 72-80 of progastrin, GRRSAE DEN (SEQ ID NO: 8).

A method of monitoring a gastrin-promoted disease or condition in a patient is also provided. The method includes determining the level of progastrin in a sample of a biological fluid from a patient suffering from or at risk of a gastrin-promoted disease or condition at a first time point; determining the level of progastrin in one or more samples of the biological fluid from the patient at one or more different time points; comparing the levels of progastrin determined at different time points and thereby monitoring the gastrin-promoted disease or condition.

The invention further provides a kit for performing an immunoassay, including an anti-progastrin-binding molecule and a suitable container. The progastrin-binding molecule selectively binds progastrin, but does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly).

Also provided is a surrogate reference standard (SRS) molecule that consists essentially of a peptide chain of from about 10 to about 35 amino acids. The SRS molecule includes immunomimics of at least two epitopes found in a protein of interest of greater than about 50 amino acids.

The invention also further provides a method of standardizing a sandwich immunoassay for a protein of interest of greater than about 50 amino acids comprising a first and a second epitope, the method comprising detecting or measuring a signal generated in the immunoassay with a standard amount of a surrogate reference standard (SRS) molecule. The SRS molecule consists essentially of a peptide chain of between from 10 to about 35 amino acids that includes immunomimics of the first and second epitopes of the protein of interest.

DETAILED DESCRIPTION OF THE INVENTION

The following provides the definitions of terms and phrases as used in this specification:

As used herein, "preprogastrin" is the 101 amino acid primary translation product of the gastrin gene and includes the N-terminal 21 amino acid signal sequence, the pro-peptide sequences and the gastrin hormone sequences.

As used herein, "Progastrin" is the 80 amino acid product formed after cleavage of the twenty-one amino acid signal sequence from preprogastrin. "Progastrin" is the primary precursor of all biologically active forms of gastrin hormone.

As used herein a "progastrin-immunomimic" is a moiety that elicits antibodies that bind progastrin and are bound by anti-progastrin antibodies. As used herein, the progastrin immunomimic moieties do not bind gastrin-17 (G17), whether amidated at the C-terminus or having a free C-terminus; glycine extended gastrin-17 (G17-Gly); gastrin-34 (G34) including both the C-terminally amidated form and the form having a free C-terminus; or glycine extended gastrin-34 (G34-Gly).

As used herein, a "gastrin hormone" or a "gastrin hormone form" are used interchangeably and mean any biologically active and/or immunologically cross-reactive gastrin hormone peptide. The major forms of gastrin hormone include, but are not limited to gastrin-17 (G17), whether amidated at the C-terminus or having a free C-terminus; glycine extended gastrin-17 (G17-Gly); gastrin-34, (G34) including both the C-terminally amidated form and the form having a free C-terminus; glycine extended gastrin-34 (G34-Gly).

A "biological fluid" as used herein means any fluid that includes material of biological origin. Preferred biological fluids for use in the present invention include bodily fluids of an animal, e.g. a mammal, preferably a human subject. The bodily fluid may be any bodily fluid, including but not limited to blood, plasma, serum, lymph, cerebrospinal fluid (CSF), saliva, sweat and urine.

A "preservative agent" as used herein means any agent, supplement or additive that reduces the time dependent degradation of gastrin in a sample of biological fluid, or a liquid sample comprising a biological component. Preservative agents useful in the practice of the present invention include any of the many preservative agents well known in the art, including but not limited to general chemical preservatives, such as for instance, sodium azide, EDTA and protease inhibitors, such as for instance, PMSF (Phenylmethylsulfonylfluoride), and aprotinin (e.g. Trasylol), or a biological preservative, such as for instance, heparin.

A "progastrin-binding molecule" as used herein may be any molecule that binds progastrin, but does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly).

A "gastrin-promoted disease or condition" as used herein means any disease or condition in which gastrin and/or progastrin has stimulatory a role. For instance, it is well known that gastrin stimulates growth and proliferation of many forms of tumors, particularly gastro-intestinal tumors, such as colorectal tumors. See U.S. 6,548,066 to Michaeli et al.

The progastrin-binding molecule of the present invention may be any progastrin-binding molecule, such as, for instance, an antibody molecule or a receptor molecule. The antibody molecule may be any antibody molecule, such as a monoclonal antibody. The anti-progastrin antibody molecules

may be single chain antibody molecules, or antibody fragments, e.g. Fab fragments, or any other antibody fragment that includes a progastrin-binding region. Preferably, the anti-progastrin antibody molecules of the invention are mammalian antibody molecules, such as rabbit, mouse or human antibody molecules. The anti-progastrin antibody molecules of the invention may be chimeric human/non-human antibodies (e.g. human/ mouse chimeras), humanized or fully human antibodies.

Monoclonal antibodies (MAbs) have unique characteristics that render them superior in many respects to polyclonal antisera and to antibodies purified from polyclonal antisera when used in many of these assays. These attributes include monodeterminant specificity for the target antigen (i.e. specificity for a single epitope), unchanging specificity among different antibody preparations, as well as unchanging affinity and chemical composition over time. Furthermore, MAbs can be produced indefinitely and in unlimited amounts by *in vitro* methods. These properties are in sharp contrast to those of polyclonal antibodies, which require *in vivo* immunization methods with the unavoidable associated biological variability and limited antibody production capacity over the lifespan of the immunized animal.

Despite these advantages, differences exist between individual MAbs even though they may be specific for the same epitope. For example, differences between MAbs induced by immunization with a single antigenic epitope region can arise with respect to any or all of the following characteristics: 1) the fine specificity for the molecular composition and tertiary structure of the epitope; 2) the antibody idioype; 3) the antibody affinity; 4) the antibody allotype; and 5) the antibody isotype. These characteristic differences can affect the behavior of MAbs in a particular immunoassay, such that different MAb isolates raised against the same antigenic region can behave differently in a given assay. Consequently, some MAbs will be superior to others that bind the same epitope when used as reagents in a particular immunoassay.

The anti-progastrin binding molecules of the present invention, especially the anti-progastrin MAbs, are particularly useful in an immunoassay. The immunoassay may be an enzyme-linked immunosorbent assay (ELISA), a radioimmunoassay (RIA), an immunodiffusion assay, or an immuno-detection assay, such as a surface plasmon resistance assay (e.g. a Biacore® assay), an ELISPOT, slot-blot, or a western blot. As a general guide to such techniques, see for instance, Ausubel et al. (eds) (1987) in "Current Protocols in Molecular Biology" John Wiley and Sons, New York, N.Y.

In a particularly useful embodiment the progastrin-binding molecules of the present invention can be used in an immunoassay such as an immunohistochemical (IHC) staining assay or an immunofluorescence (IF) procedure for visualization of a form of gastrin hormone in a tissue sample. See for example "Principles and Practice of Immunoassay" (1991) Christopher P. Price and David J. Neoman (eds), Stockton Press, New York, N.Y.

Immunohistochemical staining is of great value in detection and diagnosis of tumors, see for instance, Bodey, B. "The significance of immunohistochemistry in the diagnosis and therapy of

neoplasms" (2002) *Expert Opin Biol Ther.* 2(4):371-93. See also Osin PP, Lakhani SR. (1999) The Pathology of Familial Breast Cancer: Immunohistochemistry and Molecular Analysis. *Breast Cancer Res.* 1(1):36-40.

ANTI-PROGASTRIN MONOCLONAL ANTIBODIES

Selection of the optimal monoclonal antibody (MAb) for use in a particular application is preferably achieved by assessing the performance of each of the individual candidate MAbs in the particular intended application. For this reason, testing of candidate MAbs for optimum functionality in the intended application is part of the selective process to derive a MAb that is optimal for the intended use. This selective step is performed in addition to the selection steps normally undertaken in deriving MAbs, which include binding to the targeted antigen and serial cloning of the hybridoma that produces the MAb to ensure stability of the essential characteristics of the hybridoma cell line, including persistent cell growth and division, and consistent unlimited antibody production over an indefinite period.

As used herein, an antibody that is "selective" for a particular target epitope of progastrin means that the antibody binds the particular target epitope of progastrin with a K_a of at least about ten times higher than any other gastrin epitope, preferably one hundred times higher than any other gastrin epitope, and most preferably at least about one thousand times higher than any other gastrin epitope.

In one aspect, the present invention provides a method of identifying MAbs selective for the N-terminal and C-terminal of progastrin, wherein the MAbs have superior properties. These MAbs are particularly suitable for use in an immunoenzymometric assay (commonly termed an "ELISA" or enzyme-linked immunosorbent assay) designed to measure the particular form of gastrin hormone in a fluid, especially in a biological fluid.

The MAbs of the present invention are also suitable for detecting and/or quantifying gastrin hormone in immunodetection assays, such as for instance ELISPOT, radioimmunoassay, antibody-based sandwich capture assays, surface plasmon resistance detector systems (such as Biacore®-type systems), dot-blot, slot blot and western blot assays, as well as immunofluorescence and immunohistochemical assays.

In another aspect, the present invention provides MAbs that selectively bind progastrin at an epitope within the amino acid sequence 1-9 of progastrin (SWKPRSQQP, SEQ ID NO: 6), i.e. the product formed after cleavage of the twenty-one amino acid signal sequence from preprogastrin (the primary translation product of the gastrin gene).

In still another aspect, the present invention provides MAbs that selectively bind progastrin at an epitope within the amino acid sequence 6-14 of progastrin (SQQPDAPLG, SEQ ID NO: 7), i.e. the product formed after cleavage of the twenty-one amino acid signal sequence from preprogastrin (the primary translation product of the gastrin gene) followed by the additional removal of amino acids 1-5 of progastrin.

In a further aspect, the present invention provides MAbs that selectively bind progastrin at an epitope within the amino acid sequence 72-80 of the C-terminal region of progastrin (GRRSAEDEN , SEQ ID NO: 8) i.e. the product formed after cleavage of the twenty-one amino acid signal sequence from preprogastrin (the primary translation product of the gastrin gene).

In yet a further aspect, the invention provides MAbs that selectively bind progastrin. These MAbs bind progastrin, but do not bind the processed gastrin hormone forms: G17, G34, G17-Gly or G34-Gly. The MAbs of the invention selective for progastrin include MAbs that bind the C-terminal region of human progastrin. These MAbs that bind the C-terminal region of human progastrin also bind preprogastrin, which consists of a peptide chain of 101 amino acids from which progastrin, and gastrin are sequentially processed. However, processing of preprogastrin is rapid and occurs at the endoplasmic reticulum (ER) where it is synthesized. The MAbs of the invention that bind progastrin are useful in assays described herein to detect and quantitate progastrin in a sample.

The MAbs of the present invention can be chimerized or humanized according to established techniques well known in the art. See for instance, U.S. patent 4,816,567 to Cabilly entitled "Recombinant immunoglobulin preparations" and U.S. patent 6,689,869 entitled "Labeled humanized anti-CD-18 antibodies and fragments and kits" to Waldman et al., and U.S. patent 6,639,055 entitled Method for making humanized antibodies" to Carter et al. The humanized antibody can be reshaped to more closely match the binding affinity of the original mouse Mab. See for instance, U.S. patent 6,699,974 entitled "Re-shaped human anti-HM1.24 antibody" to Ono et al.

The present invention also provides methods for detection of progastrin samples, especially of biological samples such as biological fluids and cells, tissues, biopsy samples and organ sections etc. The invention further provides methods of diagnosis of a gastrin-promoted disease or condition in a patient by determining the presence of progastrin in diseased or in normal tissue and cells. The methods include determining the level of progastrin in a sample of a biological fluid from the patient and comparing the level of progastrin in the patient sample with the level of progastrin in a sample of biological fluid from one or more control individuals. The sample from the control individuals can be a pooled biological fluid from healthy individuals. Alternatively, the level of progastrin in the patient sample can be compared with a reference standard. The reference standard can be a standard calibrated to be within the normal range for progastrin in healthy individuals. Such control samples can readily be prepared by those of skill in the art without undue experimentation. See also below for Surrogate Reference Standards.

These methods of detection and diagnosis can be achieved by means of immunohistochemical staining of biopsy specimens using an anti-progastrin MAb of the invention. Binding of anti-progastrin MAbs to tissue samples can be visualized by immunohistochemical methods, such as, for instance, by fluorescence, immunogold or enzyme-promoted staining. For a general review of immunohistochemistry in diagnostic procedures see for example, Miller et al., Fixation and epitope

retrieval in diagnostic immunohistochemistry: a concise review with practical considerations. *Appl. Immunohistochem. Mol. Morphol.* (2000) 8(3): 228-235.

By such methods, one skilled in the art can utilize the anti-progastrin Mabs of the present invention to assess tissues, including diseased, cancerous or pre-cancerous tissue, for the presence and distribution of progastrin. This information can be useful for diagnosis and can also be helpful for selection of appropriate treatments for the diagnosed gastrin-promoted or progastrin-promoted diseases and conditions.

SURROGATE REFERENCE STANDARDS

Antibody-based assays for an antigen in which the antigen is detected or measured in an assay system that relies on binding of antibodies directed against two separate epitopes expressed by the antigen, can be limited in practical utility under certain circumstances. Such is the case when the reference antigen for the assay, against which antigen in test samples is quantified, cannot be readily obtained or synthesized. For example, in the progastrin sandwich ELISA described in the present application, a standard curve is generated using serial dilutions of a solution of reference antigen at a known concentration.

Thus, the assay to quantitate progastrin can be run to establish a standard curve using a dilution series of progastrin. This standard curve of progastrin concentration against signal produced enables quantitation of progastrin in test samples by comparison of the signal obtained with the test sample and reading the concentration of progastrin from the standard curve. The limitation of this procedure is that some antigens may be difficult, or excessively costly, to obtain in pure form and in sufficient quantity to produce a practical assay for clinical use. For example, purified progastrin (a prohormone of 80 amino acids), is costly to produce and difficult to accurately synthesize. These restrictions would severely limit the practicality of the capture ELISA for progastrin, which relies on antibodies binding to the two terminal ends of the molecule.

A solution to this problem is to substitute a Surrogate Reference Standard (SRS) in place of the native molecule. The SRS contains both of the epitopes expressed by the native molecule that are required for the immunoassay, enabling both capture and detection antibodies to bind the SRS. However, the intervening region (and/or the regions from each epitope to the end) of the native molecule is eliminated, replaced or shortened in the SRS, such that the SRS is significantly shorter than the native molecule and thus can be readily synthesized at a reasonable cost.

Peptides of up to about 35 amino acids in length can be readily and economically synthesized by existing peptide synthesis methods. Thus, the addition of a linker of from one to up to about twenty amino acids linking the two epitopes of the SRS is practical. In particular embodiments, the linker can be from about four to about fifteen amino acids in length, or from about eight to about twelve amino acids in length. Linkers can be designed to enhance assay performance by selection a linker of appropriate length and character (such as rigidity or flexibility, hydrophilicity or

hydrophobicity, and the like) for optimum performance in the particular immunoassay in which the SRS is to serve as a reference standard.

The concept of the surrogate reference standard is broadly applicable to assays wherein a simpler synthetic compound can serve as a surrogate for a more complicated natural compound. Thus, in the case of sandwich ELISAs wherein antigen is captured by antibody of one specificity and detected by antibody of a second specificity, the SRS has the general structure:

[X-epitope 1-L-epitope 2-Y]

where epitope 1 and epitope 2 are immunomimics of different epitopes of the native molecule, such that antibodies that bind epitope 1 or epitope 2 also bind the corresponding epitope of the native molecule. The SRS molecule is between about 10 and about 35 amino acids in length. Epitope 1 and epitope 2 are joined by a linker, L which may be a peptide or a non-peptide linker, or L may be a covalent bond. X and Y can each be an amino acid, a peptide sequence or a blocking group. Alternatively, either X or Y, or both can be a hydrogen atom of the N-terminus or the C-terminus, respectively, of the SRS.

Linkers useful in the practice of the present invention include any linker moiety. Such moieties are well known in the art. For instance, useful peptide linker moieties include gly-gly, as described in U.S. Pat. No. 5,759,551, issued to Ladd et al., col. 9, line 64; the inactive peptides of U.S. Pat. No. 6,613,530, issued to Wienhues et al., col. 3, lines 38-47; and the proline rich flexible hinge spacers disclosed in U.S. Pat. No. 5,683,695, issued to Shen et al. Further, non-peptide spacer moieties are also useful and have the added feature that they are generally protease resistant. Such moieties include, for instance, -O-R-CO-, -NH-R-CO-, -NH-R-NH-, -O-R-NH-, or -, -NH-R-CH₂-, in which R is a saturated or unsaturated hydrocarbon chain optionally substituted and/or interrupted by one or more aromatic radicals or heteroatoms, e.g. a nitrogen atom, an oxygen atom or a sulfur atom, as disclosed in U.S. Pat. Nos. 5,736,146, and 5,869,058, both issued to Cohen et al. The non-peptide chemical linkers disclosed in U.S. Pat. No. 6,780,969 to Wang are also useful in the practice of the present invention.

Progastrin is a large peptide of 80 amino acids that is difficult and expensive to purify in quantity. An SRS consisting of the progastrin N terminal epitope (e.g. progastrin amino acids 1-9), optionally followed by a short linker, attached to a progastrin C terminal epitope (e.g. amino acids 72-80) can be synthesized by routine methods, such as by solid phase peptide synthesis. The linker may be any linker, such as for instance an amino acid, a peptide or a non-peptide linker. The SRS is useful for standardizing a progastrin immunoassay.

In a particular embodiment, the Progastrin SRS includes two epitope-containing peptides of 9 amino acids each, optionally connected by a linker sequence between the two peptides. Shorter or longer length epitope-containing peptide sequences can be substituted, provided that the total length of the SRS does not exceed about 35 amino acid residues in length and that sufficient

binding of the antibodies to their respective cognate epitopes is retained for adequate performance of the assay.

If a peptide linker is included between the two progastrin epitopes as described above, the length of the Progastrin SRS peptide is increased by the length of the linker. For example, a linker of -Pro-Pro- or of -Gly-Gly- increases the total peptide length from 18 to 20 amino acids. Longer amino acid linkers can be substituted; the optimal sequence being selected for use as the Progastrin SRS. In a preferred embodiment the linker is a peptide that includes combinations of Proline and Glycine, such as, for instance -Pro-Pro-Gly-Gly-Pro-Pro- (SEQ ID NO: 9). Such peptides of up to about 35 amino acids in length can be readily synthesized in large quantities to a high level of purity at reasonable cost by standard methods. See for example Merrifield, *Methods in Enzymology* (1997), 289: 3-13; Also, Wade & Tragear, *Solid Phase Peptide Synthesis, Australas. Biotechnol.* (1993) 3(6):332-6.

The Progastrin SRS can be used in place of an authentic progastrin reference standard as follows. A solution of Progastrin SRS would be prepared at the same molar concentration of peptide that would be used if progastrin was the reference standard. A serial dilution of this solution is prepared, then used in the assay to establish the reference curve (Molar concentration vs. signal e.g. absorbance).

Progastrin SRS solutions can be prepared on a molar basis, providing immunometric responses identical to solutions of progastrin itself; similar, or identical standard curves are obtained with a progastrin SRS as with authentic progastrin. Absorbance values obtained from actual test samples, such as human plasma, containing progastrin, can then be compared to the SRS standard curve, to determine the concentrations of progastrin in the test samples. Comparison of the progastrin SRS standard against a progastrin standard need only be performed once and subsequently only to check accuracy.

Alternatively, the SRS can be used to generate an arbitrary standard curve that need never be calibrated against the authentic reference standard molecule. All results are then expressed as arbitrary units based by comparison with a convenient standard concentration of SRS.

The linker element may or may not be required, depending on the attributes of the SRS desired in the particular assay method in which it is to be applied. Examples of progastrin SRS peptides suitable for use in progastrin ELISAs include:

[progastrin 1-9]-[progastrin 72-80] (SWKPRSQQPGRRSAEDEN, SEQ ID NO: 10).

[progastrin 6-14]-[progastrin 72-80] (SQQPDAPLGGRRSAEDEN , SEQ ID NO: 11).

[progastrin 1-9]-Gly-Gly-[progastrin 72-80] (SWKPRSQQPGGGRRSAEDEN, SEQ ID NO: 12).

[progastrin 1-9]-Pro-[progastrin 72-80] (SWKPRSQQPPGRRSAEDEN, SEQ ID NO: 13).

[progastrin 1-9]-Pro-Gly-Gly-Pro-Pro-[progastrin 72-80] (SWKPRSQQQPGPPGRRSAEDEN, SEQ ID NO: 14).

[progastrin 6-14]-Pro-Pro-Gly-Gly-Pro-Pro-[progastrin 72-80]
(SQQPDA₆LGP₇GPPG₈P₉R₁₀S₁₁A₁₂E₁₃D₁₄N₁₅EN], SEQ ID NO: 15).

Additional potential sequences of progastrin SRS peptides can be readily determined without undue experimentation by one skilled in the art. The optimal progastrin SRS peptide for any particular immunoassay can be selected by testing candidate SRS peptides in the immunoassay system and choosing an SRS peptide that mimics authentic progastrin in the concentration range of interest under the conditions (e.g. of temperature and ionic strength, and divalent cation concentration etc.) of the assay.

PANELS OF ANTI-PROGASTRIN MONOCLONALS

The present invention provides for the first time panels of anti-progastrin and anti-gastrin MAbs that permit unequivocal identification and quantitation of progastrin and gastrin in a sample. Routine immunoassays in which the MAbs of the invention may be used include, but are not limited to, enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs), surface plasmon resistance-type assays (e.g. Biacore®-type assays), immunofluorescence assays (IFs), immunohistochemical assays (IHCs), immunodiffusion assays and the like. See for instance, U.S. patent 5,932,412 entitled "Synthetic peptides in human papilloma virus 1, 5, 6, 8, 11, 16, 18, 31, 33 and 56 useful in immunoassay for diagnostic purposes" to Dillner et al. for examples of routine diagnostic assay methods.

Supplementation of the panel of MAbs with one or more additional MAbs that selectively bind particular gastrin hormone species provides the capability of specific identification and quantitation of more than one gastrin hormone species (produced by processing of progastrin), in addition to the progastrin in a sample. For example, addition of a MAb selective for the N-terminus of the mature G17 form to the above-described panel of antibodies further permits the specific identification and quantitation of mature G17 hormone in a sample in addition to the identification and quantitation of progastrin by the anti-progastrin MAb of the present invention. Similarly, a panel of MAbs including an anti-progastrin MAb of the invention and also a MAb selective for the N-terminus of G34 also allows the specific identification and quantitation of G34 in a sample. Thus, as illustrated above, addition to the panel of a MAb selective for any particular gastrin hormone form can be used to supply further information as to the nature and amounts of the particular gastrin hormone form in the sample in addition to the information related to progastrin obtained with the anti-progastrin MAb of the invention.

Other combinations of MAbs useful in a panel of MAbs for identification, quantitation and monitoring of other forms of gastrin hormone will be immediately apparent to those of skill in the art. The present invention encompasses all such pairs of MAbs of the invention and combinations of two or more of MAbs of the invention.

The MAbs of the present invention provide the means to accurately determine the amounts and ratios of progastrin/gastrin hormone forms for assessment of predispositions to gastrin-hormone-

promoted diseases and conditions, and for detection and diagnosis of such diseases and conditions in patients suffering therefrom. For example, the anti-gastrin MAbs of the invention can be incorporated into ELISA assays for large scale screening of patient serum or other biological fluid for progastrin and any one or all of the G17, G34, and the G17-Gly, and G34-Gly gastrin hormone forms.

The MAbs of the present invention, combinations of pairs of MAbs selected from the MAbs of the invention, and panels of MAbs of the present invention are particularly useful when applied to high-throughput methods. Such methods include micro-chip and micro-array methods of gastrin hormone antigen detection, such that many samples can be tested on a microplate or slide, or other assay substrate, such as a plate with virtual wells (such as for instance, that described in U.S. patent 6,565,813 to Garyantes et al). Detection of binding can be by any one of the many state-of-the-art detection systems currently available. Detection of binding can be, for instance, by surface plasmon resistance (SPR) changes caused by specific biomolecular reactions, such as antigen-antibody binding. See also, for example, U.S. patent 5,981,167 to Taremi et al. for an application of this technology to enzymatic assays. The technology may be applied in a continuous flow mode and is equally applicable to detection of antibody binding to a surface-immobilized peptide or protein, such as a gastrin hormone, or to the detection of a gastrin-antibody complex. The latter complex may be detected by binding to a surface immobilized antibody specific for an epitope of the form of gastrin hormone (G17, G34, G17-Gly or G34-Gly) binding to which is not sterically hindered by the antibody of the complex. Furthermore, this technology has the advantage of high throughput applicability and high sensitivity without the requirement for a radiolabel.

The MAbs of the present invention are also useful for immunohistochemical (IHC) and immunofluorescence (IF) assays of tissue samples, such as for instance, from biopsy material. Such analyses can be used to detect aberrant levels of individual gastrin-hormone forms and hence to diagnose gastrin-hormone-promoted diseases and conditions.

The MAbs of the present invention are also useful for prevention, diagnosis and therapy of progastrin-hormone-promoted diseases and conditions. The anti-progastrin MAbs of the invention can be formulated in pharmaceutical compositions for passive immunization against particular gastrin hormone forms. See for example, U.S. patent 6,391,299 (herein after the '299 patent) entitled "Anti-factor IX/IXa antibodies" to Blackburn et al. Functional fragments of the MAbs of the present invention, such as, for instance Fab fragments, F(ab')₂ fragments and any fragments (see the '299 patent for fragment descriptions) that retain the ability to bind the gastrin hormone form to which they are directed can also be incorporated into pharmaceutical compositions and applied in therapy. See the '299 patent for useful pharmaceutical compositions. The preferred routes of administration of the pharmaceutical compositions of the invention include parenteral routes of administration, such as subcutaneously, intramuscularly and intravenously. Additionally, the oral route can be used for therapy of certain diseases, particularly diseases of the gastrointestinal tract, such as ulcerative diseases of the esophagus or stomach.

Alternatively, the pharmaceutical compositions of the present invention can be delivered intranasally. Such pharmaceutical compositions are particularly useful when administered in an effective amount for the prevention or therapy of gastrin-hormone-promoted diseases or conditions in patients having a prognosis of high likelihood of such diseases or conditions, treatment of patients already suffering from such diseases or conditions. The pharmaceutical compositions of the invention are also useful for the alleviation of symptoms and the arrest of progression of progastrin-promoted diseases and conditions.

An effective amount of a pharmaceutical composition that includes an intact or functional fragment of an anti-gastrin MAb, particularly a humanized anti-gastrin MAb of the invention for the treatment of a gastrin-promoted disease or condition is defined as an amount that prevents onset of or reduces the rate of progression of the disease or condition; more preferably an effective amount is an amount that stabilizes the disease or condition; more preferably still an effective amount is an amount that causes regression of the disease or condition. Most preferably, an effective amount is an amount that completely cures the disease or condition.

Furthermore, the MAbs of the present invention can be applied in immunoassays for monitoring the progression of gastrin-hormone-promoted diseases and conditions, where the level or amount of progastrin provides an indication of the success of treatment or therapy, or of progression of the disease or condition.

Moreover, the MAbs of the present invention are useful in methods of evaluating a progastrin hormone blocking treatment of a patient suffering from a gastrin hormone-promoted disease or condition. The method includes the steps of:

- a) obtaining a first sample of biological fluid from the patient prior to or in the early stages of a treatment;
- b) determining the level of progastrin in the first sample by an immunoassay method;
- c) obtaining a second sample of biological fluid from the patient after a suitable time within which the treatment would have an effect;
- d) determining the level of progastrin in the second sample by the immunassay method,
- e) comparing the determined amounts of progastrin in the first sample with the amount of progastrin in the second sample so as to determine the efficacy of the progastrin binding or blocking treatment.

The above-described method applied to evaluating a progastrin binding treatment or blocking treatment in a patient is particularly valuable in clinical practice, where timing of decisions to proceed with one therapeutic regimen or another may be critical to the outcome for the patient. The method of the present invention provides information on which to base these critical decisions. The method provides a measurement of the progastrin amount prior to or in the early stages of treatment and provides one or more measurements of progastrin at one or more periods after initiation of treatment, particularly when the treatment is expected to have begun to be effective.

The progastrin blocking treatment may be passive administration of anti-progastrin antibody to a patient. The progastrin blocking substance may any progastrin blocking substance, including but not limited to an anti-progastrin antibody, particularly a chimeric human/non-human antibody, a humanized or a fully human monoclonal anti-progastrin antibody, or any other molecule that is functional in binding progastrin.

The present invention also provides compositions, methods and kits for screening samples suspected of containing progastrin. Such screening may be performed on patient samples, or laboratory samples suspected of containing or producing such a polypeptide. A kit can contain an antibody of the present invention. The kit can contain a suitable buffer and reagents for detecting an interaction between a sample and antibody of the present invention. The provided reagent can be radiolabeled, fluorescently-labeled or enzymatically-labeled agent capable of binding or interacting with an antibody of the present invention.

The reagent of the kit can be provided as a liquid solution, attached to a solid support or as a dried powder. When the reagent is provided in a liquid solution, preferably, the liquid solution is an aqueous solution. Preferably, when the reagent provided is attached to a solid support, the solid support can be chromatographic media, a test plate having a plurality of wells, or a microscope slide. When the reagent provided is a dry powder, the powder can be reconstituted by the addition of a suitable solvent, which may be provided.

The kit of the invention is provided in a container that generally includes a vial into which the antibody, antigen or detection reagent may be placed, and preferably suitably aliquotted. The kits of the present invention will also typically include a means for containing the antibody, antigen, and reagent containers for commercial sale. Such containers may include plastic containers into which the desired vials are retained and one or more necessary chemicals, such as chromatography material, solvents and eluents, test tubes, detergents, antibodies and chemicals for the detection reaction.

In still further embodiments, the present invention concerns immunodetection methods and associated kits. It is proposed that progastrin or peptide fragments thereof may be employed to detect antibodies having reactivity therewith, or, alternatively, antibodies prepared in accordance with the present invention, may be employed to detect progastrin, progastrin-mimic molecules or progastrin epitope-containing peptides. In general, these methods will include first obtaining a sample suspected of containing such a precursor, peptide or antibody, contacting the sample with an antibody or peptide in accordance with the present invention, under conditions effective to allow the formation of an immunocomplex, and then detecting the presence of the immunocomplex.

A wide variety of methods of detection of immunocomplex formation are well known in the art, for example, ELISA, RIA, immunoblot (e.g., dot blot, slot blot, western blot etc.), indirect immunofluorescence techniques and methods that rely on detection of changes in physical parameters, such as for instance, surface plasmon resistance and the like. In one widely used

method immunocomplex formation is detected through the use of a label, such as a radiolabel or an enzyme tag (such as alkaline phosphatase, or horseradish peroxidase). Additional advantages may accrue through the use of a secondary binding ligand such as a second antibody or an avidin-coupled molecule for binding a biotinylated ligand, according to methods well known in the art.

EXAMPLES

EXAMPLE 1. Production of Mabs to the N-terminal end of Human Progastrin.

The peptide, SWKPRSQQPPC ("hProGastrin (1-9)-PC", SEQ ID NO: 16) containing the amino acid sequence 1-9: SWKPRSQQP (SEQ ID NO: 17) of human progastrin, forming the N-terminal end epitope of human progastrin, followed by the linker sequence -Pro-Cys-, was synthesized commercially by standard solid phase peptide synthesis methodology.

The peptide was incorporated into an immunogen to induce antibodies to the N-terminus of human progastrin as follows: The peptide was first covalently linked to diphtheria toxoid ("DT") to yield a peptide-carrier conjugate. The number of peptide units substituted on each DT carrier was determined and finally, the conjugate was formulated as an immunogen. The techniques used were as described in U.S. Patent 5,622,702 to Gevas et al.

Briefly, the peptide was chemically conjugated to the carrier with the heterobifunctional cross-linker, epsilon-maleimidocaproic acid N-hydroxysuccinimide (ϵ -MCS). The conjugate was purified by dialysis against 0.1M sodium phosphate buffered saline, pH 7.3 (PBS) and the protein concentration determined by the Lowry assay. The substitution level of peptide on DT (14.7 peptides per 100,000 Da molecular weight of DT) was determined on a molar basis by amino acid analysis of the conjugate. The dissolved conjugate was then formulated as an immunogen with Montanide ISA 703 (SEPPIC, France) as adjuvant by mixing the conjugate solution with the Montanide ISA 703 oil at a 30/70 ratio (wt/wt of conjugate solution/adjuvant). Mixing was achieved by drawing the appropriate volumes of each liquid into a syringe and then rapidly passing the solutions back and forth between this and a second syringe through an inter-locking hub.

Mice were initially immunized by i.p. injection with 0.1 mg of the peptide-DT conjugate immunogen/Montanide ISA 703 in a volume of 0.1 mL. A second injection of an identical dose was given three weeks after the first injection.

To create hybridomas producing a MAb selective for the N-terminus of human progastrin, spleen cells from the immunized mice were fused with a standard mouse myeloma fusion partner cell line by standard techniques well known to those skilled in the art. See, for instance, U.S. patent 4,196,265 *Method of producing antibodies* to Kaprowski et al; "Selected Methods in Cellular Immunology" (Chapter 17: *Immunoglobulin Producing Hybrid Cell Lines*, B. Mishell and S. Shiigi, W.H. Freeman and Co., San Francisco, 1980); Harlowe and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1988; Zola, *Monoclonal Antibodies: A Manual of Techniques*, CRC Press, Inc., Boca Raton, FL, 1987. Immunized mice were boosted with an i.p. injection of 0.1 mg of the above-described peptide-DT conjugate in PBS 4 days prior to collection

of their spleen cells for the cell fusion. Initial selection of hybrid cells was done using hypoxanthine-aminopterin-thymidine supplemented media, as described in Mishell and Shiigi. This fusion was designated F490.

The first selection step for isolating hybridomas producing MAbs to the N-terminal end of human progastrin was selection of cells for production of antibody to the target peptide and for stability of the hybrid cell lines. The selection of cells producing antibody was accomplished by screening cell culture media obtained from tissue culture wells containing single clones for antibody to the N-terminal end of human progastrin. The screening was by means of an ELISA using as target antigen a conjugate of the synthetic peptide hProGastrin(1-9)-PC linked through the terminal cysteine-11 to bovine serum albumin (BSA) as an immunological carrier. Suitable ELISA techniques are known to those skilled in the art, several examples of which are listed below. Stable cell lines were obtained by twice cloning each hybrid that produced antibodies that bound the hProGastrin(1-9)-PC-BSA conjugate in the ELISA test. By these methods, eight hybrid cell lines were obtained that produced MAbs to the N-terminal end of human progastrin. These hybrid lines were designated: 490-1; 490-2, 490-3; 490-4, 490-5; 490-6, 490-7 and 490-8.

EXAMPLE 2. Production of MAbs to the N-terminal region of Human Progastrin.

The peptide, SQQPDAPLGPPC ("hProGastrin(6-14)-PPC". SEQ ID NO: 18) containing the amino acid sequence 6-14 SQQPDAPLG (SEQ ID NO: 19) of human progastrin, forming an N-terminal region epitope of human progastrin, followed by the linker sequence -PPC, was synthesized commercially by standard solid phase peptide synthesis methodology.

The SQQPDAPLGPPC peptide (SEQ ID NO: 18) was incorporated into an immunogen to induce antibodies to the N-terminal region of human progastrin as described in Example I above. Immunization and isolation of a fusion designated F491 was also performed as described above.

The selection of hybridomas producing MAbs to the N-terminal region of human progastrin was accomplished by screening cell culture media obtained from tissue culture wells containing single clones for antibody to the N-terminal region of human progastrin. Screening was by an ELISA using as target antigen a conjugate comprising the synthetic peptide hProGastrin(6-14)-PPC, linked through cysteine-12 to bovine serum albumin (BSA) as an immunological carrier. Stable cell lines were obtained by twice cloning each hybrid that produced antibodies that bound the hProGastrin(6-14)-PPC-BSA conjugate in the ELISA. Three hybrid cell lines were obtained that produced MAbs to the N-terminal region of human progastrin. These hybrid cell lines were designated: 491-1, 491-2 and 491-3.

EXAMPLE 3. Production of MAbs to the C-terminal end of Human Progastrin.

The peptide, CPPGRRSAEDEN ("hProGastrin(72-80)-PPC". SEQ ID NO: 20) having the amino acid sequence 72-80 GRRSAEDEN (SEQ ID NO: 21) of human progastrin at the C-terminal end preceded by the linker sequence CPP-, was synthesized commercially by standard solid phase

peptide synthesis methodology. The peptide was incorporated into an immunogen to induce antibodies to the C-terminus of human progastrin as described in Examples 1 and 2 above.

Mice were initially immunized by i.p. injection with 0.1 mg of the peptide-DT conjugate immunogen/Montanide ISA 703 in a volume of 0.1 mL. A second injection of an identical dose was given three weeks after the first injection.

To create hybridomas producing a MAb selective for the C-terminus of human progastrin, spleen cells from the immunized mice were fused with a standard mouse myeloma fusion partner cell line by standard techniques as described above to produce the fusion designated F495.

The first selection step for isolating hybridomas producing MAbs to the C-terminal end of human progastrin was selection of cells for production of antibody to the target peptide and for stability of the hybrid cell lines. The selection of cells producing antibody was accomplished by screening cell culture media obtained from tissue culture wells containing single clones for antibody to the C-terminal end of human progastrin. The screening was accomplished by means of an ELISA using as target antigen a conjugate comprising the synthetic peptide hProGastrin(72-80)-PPC, linked through cysteine-1 to bovine serum albumin (BSA) as an immunological carrier. Stable cell lines were obtained by twice cloning each hybrid that produced antibodies that bound the hProGastrin(72-80)-PPC-BSA conjugate in the ELISA. Four hybrid cell lines were obtained that produced MAbs to the C-terminal end of human progastrin. These hybrid lines were designated: 495-1; 495-2, 495-3 and 495-4.

EXAMPLE 4. ELISA Titors of Antibodies to specific regions of progastrin

The purpose of this analytical method is to detect and to determine the titer of anti-progastrin antibodies in test samples by ELISA. The anti-progastrin antibody ELISA of the invention is based upon the specific binding of antibodies (which may be polyclonal antibodies or monoclonal antibodies) to a progastrin peptide-BSA conjugate. Binding of the peptide in the ELISA confirms that the antibody specifically binds a progastrin epitope within the progastrin peptide sequence of the conjugate.

The progastrin-BSA conjugates tested included the three conjugates listed in Examples 1, 2 and 3: hProGastrin(1-9)-PC-BSA; hProGastrin(6-14)-PPC-BSA; and hProGastrin(72-80)-PPC-BSA (respectively). The conjugates were prepared using the same progastrin peptides used to make the DT-linked immunogens, using the cross-linking reagent ϵ -MCS, as described above.

In the first step of the ELISA, conjugate was bound to the wells of a 96 well ELISA plate. Free conjugate was removed by a wash step using a 96 well plate washer. The test (or control) antiserum was then added. Anti-progastrin Ab present in the test serum bound to the conjugate by virtue of progastrin peptide epitopes present on the antigen. Bound antibodies were then detected by the addition of an anti-IgG-Alkaline Phosphatase reagent, which is species specific for the anti-progastrin antibodies being detected. For example, mouse anti-progastrin antibodies were detected using Rabbit anti-Mouse IgG-Alkaline Phosphatase conjugate ("RAM-AP"), which binds to the

mouse anti-progastrin Ab, as the Ab detection reagent. The AP moiety of anti-Ig-AP conjugate subsequently catalyses conversion of substrate to a colored product (p-nitrophenol). Color development was measured as absorbance at 405 nm in an ELISA plate reader.

Pooled mouse anti-progastrin serum (collected from the mice immunized for the production of anti-progastrin hybridomas) or ascites fluid containing mouse anti-progastrin MAb was used as positive control for ELISAs targeting the progastrin epitope that was used to induce the antibodies. Serum from the same animal species as the test sample was used as negative control (e.g., normal sera, or pre-immune sera, etc.).

The magnitude of color development in the linear range was directly proportional to the quantity of anti-progastrin Ab bound to the target antigen. A plot of the dilution series of the positive standard (anti-progastrin) serum versus absorbance values was used to generate binding curves. The anti-progastrin Ab titers of the test samples were then determined from the greatest dilution that produces an absorbance that can be distinguished from the absorbance obtained with the same dilution of negative control (limiting dilution analysis).

REAGENT SOLUTIONS: The quantities of reagents and solutions specified for preparation in this analytical method were chosen for convenience only and are provided as examples, and are not to be taken as limitations. The actual quantities can be scaled according to requirements.

1. Carbonate buffer with 0.02 % NaN₃ ("Carbonate buffer") was prepared by dissolving 1.59 g Na₂CO₃ and 2.93 g NaHCO₃ in approximately 750 ml of distilled water and stirring with a magnetic stirrer. 4 ml of 5 % NaN₃ solution was added and stirred. The solution was adjusted to 1.0 liter with water. The pH was measured, (this should be 9.6 ± 0.2) and adjusted with 1.0 M NaOH or 1.0 M HCl if necessary). The buffer was stored in a refrigerator until needed.
2. FTA (PBS) with 0.05 % Tween-20 and 0.02 % NaN₃ ("FTA/Tween") was prepared by dissolving 9.23 g FTA in approximately 750 ml of purified water. 0.5 ml Tween-20 and 4 ml 5% NaN₃ was added and adjusted to 1.000 liter with water.
3. 1 % BSA in FTA/Tween ("BSA/FTA/Tween") was prepared by dissolving 10 g BSA in 1000 ml FTA/Tween.
4. Substrate buffer: 50 mg MgCl₂ • 6H₂O was dissolved in 448 ml of purified water. 50 ml of DEA and 2 ml 5% NaN₃ were added and the pH adjusted to 9.8 with concentrated HCl. The buffer was stored protected from light at room temperature.
5. PBS, pH 7.2: Was prepared from solid FTA (FTA Hemagglutination Buffer ("FTA") (Becton Dickenson Microbiology Systems, Cockeysville, MD)).

ELISA PROCEDURE: Coating with Antigen: A solution of 1 µg/ml progastrin-BSA conjugate target antigen (described above), in Carbonate buffer was prepared. A minimum of 5.2 ml of antigen solution was needed for each plate to be coated. Antigen solution is prepared by making a 1:1000 dilution of the 1 mg/ml conjugate stock solution with Carbonate buffer. Plates may be any

plate suitable for ELISA assays, such as for instance, Microtiter® Immunoassay Plates, rigid styrene (e.g., Immulon® 2 "U" bottom 96 well plates, Dynatech Laboratories, Inc., VA; or Flat-bottom 96 well plates, polystyrene: e.g., Microwell Plates, NUNC, vendor VWR). Immulon® 2 "U" bottom plates are coated with antigen by adding 50 µl/well of the antigen solution. Plates were stored in a moist chamber (e.g., a closed container with a moist paper towel) to prevent moisture loss and incubated overnight in the refrigerator (at 2°-8°C).

PREPARATION OF SERUM DILUTIONS: Any convenient dilution series is acceptable. For example, 1/10^{0.5} serial dilution series of the positive standard and negative control and test sera were used as shown in Table 1. Samples were diluted in BSA/FTA/Tween solution in flat bottom 96 well plates (12-channel multipipettors enable simultaneous dilution of up to 12 sera).

TABLE 1 Serial dilutions starting at 1:1000 were prepared as shown

96 well plate	Serum	Titer ¹
Row #	Dilution	(= 1/Dilution)
A	1:1,000 = 10 ⁻³	10 ³
B	1:3,162 = 3.16 x 10 ⁻⁴ = 10 ^{-3.5}	3.16 x 10 ³
C	1: 10,000 = 10 ⁻⁴	10 ⁴
D	1: 31,623 = 3.16 x 10 ⁻⁵ = 10 ^{-4.5}	3.16 x 10 ⁴
E	1: 100,000 = 10 ⁻⁵	10 ⁵
F	1: 316,230 = 3.16 x 10 ⁻⁶ = 10 ^{-5.5}	3.16 x 10 ⁵
G	1: 1,000,000 = 10 ⁻⁶	10 ⁶
H	1: 3,163,300 = 3.16 x 10 ⁻⁷ = 10 ^{-6.5}	3.16 x 10 ⁶

¹. The titer of each dilution is calculated as the reciprocal of the dilution.

A sufficient volume of a dilution of each sample was prepared to provide a minimum working volume of 200 µl. Depending on the sample titer, dilutions beginning with a 1/100 (for low titer sample) or 1/1000 (for high titer sample) dilution of each sample in row A were made, then proceeding with serial dilutions down each column to row H (See Table 9), yielding a total of eight dilutions of each sample. The dilution series of the negative control was prepared beginning at 1/100. Samples of the dilution series of the positive standard antibody and the prebleed/negative control antibody were run in duplicate on each plate.

PLATE WASHING: Using the plate washer, (e.g., Ultrawash Plus; or, DynaWasher II (Dynatech Laboratories, Inc., VA) or equivalent) the coated plates were washed four times each with FTA/Tween and then "slapped" the plates on paper towels to remove residual solution.

ANTIBODY BINDING: Following the sample plate dilution series as shown in Table 2 below, 50 µl/well of the diluted sample was transferred to the corresponding wells of the antigen coated "U" plates. The plates were incubated in a moist chamber for 1 hour at room temperature.

TABLE 2: EXAMPLE OF A 96 WELL PLATE ELISA SETUP

	1	2	3	4	5	6	7	8	9	10	11	12	Sample Dilution
A	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	10^{-3}
B	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	$10^{-3.5}$
C	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	10^{-4}
D	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	$10^{-4.5}$
E	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	10^{-5}
F	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	$10^{-5.5}$
G	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	10^{-6}
H	Neg.	Neg.	Pos.	Pos.	TS1	TS2	TS3	TS4	TS 5	TS 6	TS 7	TS 8	$10^{-6.5}$

Abbreviations:

Pos. = Positive standard sample;

Neg. = Prebleed/negative control sample;

TS 1-TS 8 = Test samples

ANTIBODY DETECTION REAGENT: An appropriate dilution of Anti-Ig-Alkaline Phosphatase Conjugate was prepared in FTA/Tween. A minimum of 5.2 ml per plate in the assay was required. Plates were washed as described above. 50 µl/well of the RAM-AP solution (Anti-Ig-Alkaline Phosphatase Conjugate e.g., for testing mouse anti-progastrin antibodies, Rabbit anti- Mouse IgG (H+L)-Alkaline Phosphatase (Zymed)) was added to every well in the "U" plate and incubated at room temperature in the moist chamber for 1 hour.

To detect anti-progastrin antibodies in serum obtained from species other than mouse, an anti-Ig-AP conjugate must be used that is specific for the species that produced the test serum (e.g., human anti-progastrin antibodies are detected with an anti-human IgG-AP reagent, used at the dilution established for each lot of reagent). The positive standard and negative control serum should be obtained from the same species as the test serum.

SUBSTRATE SOLUTION: p-NPP tablets (p-nitrophenylphosphate, supplied as Phosphatase Substrate Tablets, Sigma 104 ("p-NPP") (Sigma Chemical Co., St. Louis, MO)) were removed from the freezer and allowed to warm to room temperature. Immediately before use, a 1 mg/ml solution of p-NPP was prepared by adding one tablet of p-NPP to 5 ml of DEA substrate buffer at room temperature. Each 5-ml aliquot of substrate solution was sufficient for one assay plate. Substrate solution was stored in the dark until used.

SUBSTRATE ADDITION: Plates were washed as described above. To all wells, beginning with column 1, 50 μ l/well of p-NPP solution was simultaneously added with an 8 (or 12) channel multipipettor beginning with row A.

MONITORING REACTION: The development of the substrate solution was stopped after 10-15 minutes.

STOP REACTION: The reaction was stopped by adding 50 μ l of 1.0 M NaOH to each well with the 8 (or 12) channel pipettor. The NaOH solution was added to the wells in the same order, and with the same timing, as the substrate solution was added. The reagents were gently mixed by carefully shaking the plate on the counter top.

MEASURE ABSORBANCE: The entire plate was read with an ELISA reader. The ELISA reader was set to measure at $A_{405\text{ nm}}$ for p-nitrophenol.

DATA ANALYSIS: The antibody titer of each serum was determined as follows: The absorbance obtained for each sample was plotted on the ordinate (linear scale) against (1/dilution) on the abscissa (log scale) for each serum, including the positive standard, on a semi-log graph scale. By plotting the inverse of the dilution, the titer could be read directly on the X-axis. Occasionally, an absorbance value was clearly off the binding curve for a particular serum (outlier points); such values were excluded from the curve. The titer of each serum is determined as the final dilution of test antibody that can be distinguished from the same dilution of negative control sample, based upon absorbance values. Generally, the limit of differentiation between the two results is an absorbance of 0.25 absorbance units or more (depending on sample to sample variability in the assay).

EXAMPLE 5. Determination of Antibody Specificity by Inhibition ELISA

The same method as in the Example above is followed for the peptide inhibition ELISA with the exceptions described below.

PREPARATION OF INHIBITOR: The appropriate target hormone peptide, in this case peptide(s) expressing progastrin epitope(s), were prepared as working stocks of 1 μ mol/ml (1000 μ M). The inhibition dilution series was prepared from the working stock solution, at dilution ratios from 1:2 to 1:10, yielding a total of 8 or 12 dilutions depending on the layout on the plate.

Preparation of Sample Dilution: A series of titrations of the sample were done prior to the inhibition assay to establish the dilution of the antibody sample at 50% maximal binding. The

sample was then prepared to 2X the 50% binding concentration, for mixing with equal volumes of peptide inhibitor and with buffer as a control in the inhibition assay. The sample mixture was incubated in a moist chamber for approximately 30 minutes and then added to the washed coated ELISA plate and incubated for approximately 1 hour in a humidity chamber. The percent binding was determined from the absorbance readings (subtracted from the background) by dividing the absorbance obtained from the sample with inhibitor by the absorbance obtained from the sample control without inhibitor, and multiplying this value by 100. Finally, the percent inhibition was determined by subtracting the percent binding from 100%.

The test samples for use in this assay can be serum, MAb in cell culture supernatant, ascites fluid, or affinity-purified antibody (Ab). For Abs against target antigens other than the amino terminus of progastrin, the appropriate target hormone antigen and inhibitor are used. An unrelated peptide inhibitor should be included as a negative control.

EXAMPLE 6. Anti-progastrin MAb for Isotype and Specificity by ELISA

The anti-progastrin MAb described in Example 1 (F490), Example 2 (F491) and Example 3 (F495) were characterized for isotype and specificity by ELISA. The target antigen for each MAb was the same as that described in Examples 1 through 3. Thus, MAb from fusion 490 were tested against hProGastrin(1-9)-PC-BSA; MAb from fusion 491 were tested against hProGastrin(6-14)-PPC-BSA; and, MAb from fusion 495 were tested against hProGastrin(72-80)-PPC-BSA. Isotypes were determined by the method of Example 4, wherein the secondary rabbit anti-mouse Ig reagents were specific for mouse antibody isotypes. Specificity was determined by the method of Example 5, using the following peptides as inhibitors: hProGastrin(1-9)-PC, hProGastrin(6-14)-PPC, hProGastrin(72-80)-PPC, human G17, human G34, human G17-Glycine extended and luteinizing hormone releasing hormone (LHRH) as a negative control.

The results of these tests are given in Table 3. As the Table shows, all of the MAb were of the IgG1 subclass, with the exception of antibodies 491-2 and 495-2, both of which were of the IgG2a subclass. The Table also shows that each of the individual MAbs was specific for the epitope used for immunization. Thus, all of the 490 series MAbs were specific for the progastrin epitope sequence 1-9; all of the 491 series MAbs were specific for the progastrin epitope sequence 6-14; and, all of the 495 series MAbs were specific for the progastrin epitope sequence 72-80. These MAb are deemed suitable for tests designed to detect and measure progastrin in biological test samples consisting either of fluids (e.g., plasma, lymph, ascites, saliva, etc.) and in tissue specimens, (e.g., biopsy specimens of normal tissue or tumor origin, or of shed cells from such tissues, etc.).

TABLE 3: Characterization of MAbs from Fusions 490, 491 and 495.

Specificity	MAb	Endpoint	Antibody Subclass	Percent Inhibition with 25 micromolar Peptide						
				ProG 1-9	ProG 6-14	ProG 72-80	G17	G17-gly	G34	LHRH
Progastatin 1-9 (N terminal end)	490-1	30,000	IgG1	100	0	0	0	0	0	0
	490-2	30,000	IgG1	100	0	0	0	0	0	0
	490-3	30,000	IgG1	100	0	0	0	0	0	0
	490-4	30,000	IgG1	100	0	0	0	0	0	0
	490-5	10,000	IgG1	100	0	0	0	0	0	0
	490-6	10,000	IgG1	100	0	0	0	0	0	0
	490-7	10,000	IgG1	100	0	0	0	0	0	0
	490-8	10,000	IgG1	100	0	0	0	0	0	0
Progastatin 6-1 (N term region)	491-1	>30,000	IgG1	0	100	0	0	0	0	0
	491-2	10,000	IgG2a	0	100	0	0	0	0	0
	491-3	1,000	IgG1	0	100	0	0	0	0	0
Progastatin 72-80 (C terminal end)	490-1	30,000	IgG1	0	0	100	0	0	0	0
	490-2	30,000	IgG2a	0	0	100	0	0	0	0
	490-3	30,000	IgG1	0	0	100	0	0	0	0
	490-4	10,000	IgG1	0	0	100	0	0	0	0

EXAMPLE 7. Synthesis of Progastatin Surrogate Reference Standards (SRS) for Measurement of Progastatin by Immunoassay

To demonstrate the utility of SRS, two progastatin SRS peptides were synthesized by solid phase peptide synthesis. The synthesis methods employed were those in common commercial usage and are well known to those skilled in the art.

Progastatin SRS 1 was synthesized for use in an assay to detect and quantify human progastatin 1-80. The progastatin SRS 1 peptide had the following structure: [progastatin 1-9 – (PGGPP) – progastatin 72-80]. The amino acid sequence of the peptide was: SWKPRSQQQPGGGPPGRRSAEDEN (SEQ

ID NO: 14). The mass of this peptide was 2535.1, and the purity of this peptide was greater than 90% when tested by HPLC.

Progastrin SRS 2 was synthesized for use in an assay to detect and quantify human progastrin 6-80. The progastrin SRS 2 peptide had the following structure: [progastrin 6-14 – (PPGGPP) – progastrin 72-80]. The amino acid sequence of the peptide was: SQQPDAPLGPPGGPPGRRSAEDEN (SEQ ID NO: 15). The mass of this peptide was 2432.4, and the purity of this peptide was greater than 90% when tested by HPLC.

EXAMPLE 8. Immunoenzymometric Assay Utilizing Anti-Progastrin MAb and Progastrin SRS Peptides to Measure Progastrin

The following analytical methods (immunoenzymometric assay) were used to determine free (non-complexed) human progastrin 1-80 or human progastrin 6-80 present in biological fluids such as human plasma, by using monoclonal and/or polyclonal antibodies directed to the N-terminus or the C-terminus of the particular molecular form of progastrin that is being assayed. Alternatively, a combination of a polyclonal antibody directed to the N-terminus or to the C-terminus of the molecule can be used in combination with a monoclonal antibody directed to the C-terminus or to the N-terminus of the molecule respectively.

1. Plate Coating: Antibody selective for the N terminus of the particular human progastrin molecular form to be tested was coated at an optimal concentration onto the surface of the micro wells of a test plate. NUNC Maxisorp, F 96 ELISA plate (cat. No. 439454) test plates were used and the antibody coating solution is prepared in sodium borate buffer (20mM, pH 8.0, containing 0.1% sodium azide). The concentration of affinity purified Mab in the coating solution was preferably about 5 µg/mL. 100 µL of Mab solution is added per well, and coating was allowed to proceed overnight at 4°C in a humidified sealed box. Plates were coated with affinity purified 490-1 Mab for the detection of progastrin 1-80, using SRS 1 to establish a standard curve in the assay. Plates were coated with affinity purified 491-1 Mab for the detection of progastrin 6-80, using SRS 2 to establish a standard curve in the assay.

2. Plate washing: The coating solution were removed and wash buffer (approx. 400 µl per well) was added and then removed. This wash cycle was repeated as many times as required; generally, three or four washes total. Wash buffer was 0.010 M phosphate buffer; 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4, containing 0.01% w/v Triton X-100). Plate washing may be automated.

3. Plate blocking: Blocking buffer containing protein and detergent (1% BSA/0.1% Triton X-100 in coating buffer) was added to the micro wells (200 µL/well), and the plates were incubated for 1 hour at room temperature in a humidified box. Plates were stored refrigerated at approximately 4°C in this form.

4. Sample and standard addition: Plates were washed as described above. Reference standards (such as, for instance, SRS 1, SRS 2, or authentic progastrin forms; or, negative control peptide

such as Gastrin 17) were prepared as dilution series to generate standard curves. In the tests of this example, SRS 1 and 2 were prepared at a concentration of 10 μ M, and diluted to 100 fM in 1:10 dilution series. The standards and test samples were prepared in assay buffer (1% BSA, 0.1% bovine γ -globulin prepared in wash buffer). The solutions were then added to each well (100 μ L/well). The reaction was allowed to proceed for 2 hours at room temperature in a humidified box.

The optimal antigen concentration was determined by generating a standard curve using known concentrations of progastrin SRS of the form to be assayed, the standard curve having the required sensitivity and precision over the required useful concentration range. For progastrin (either 1-80 or 6-80), the useful progastrin concentration range of the assay was generally from background (about 1 pM or less) to about 100 nM. Greater accuracy can be achieved by using a narrower dilution series of reference standard, such as a 1:2 dilution series.

It will be immediately recognized that assay sensitivity and precision can be readily modified or enhanced by alteration of other assay parameters, such as the selection of the particular Mab for well coating or for enzyme labelling, the concentrations of reagents, the composition of buffers, the selection of enzyme-substrate systems, the incubation times, and other parameters that can be modified to suit the requirements of the assay. The appropriate sensitivity and precision over the required range can be readily determined by those of ordinary skill in the art without undue experimentation.

5. Addition of conjugate: Following washing, assay buffer containing monoclonal or polyclonal antibody specific for the C-terminus of the progastrin form to be assayed (conjugated with an enzyme label), was added to each well. In the case of this example, MAbs against the C-terminal end of progastrin were affinity purified then coupled to horse radish peroxidase (HRP). The MAbs were shown to retain binding to the C terminal end of progastrin and to have HRP activity. Separate conjugates were made with each of the four MAbs 458-1 through 458-4. For the purposes of this test, the conjugates were used separately and not mixed. The conjugates were used at a 1:2000 dilution of the stock solution of 630 μ g/mL, and 100 μ L was added per well. The reaction was allowed to proceed at room temperature (nominally +22 °C) for at least 1 hour.

6. Addition of substrate: The wells were washed as described above, and 100 μ L of TMB Solution (Pierce) substrate solution was added to each well. Reactions were allowed to proceed for 30 minutes, then 100 μ L of stop solution of 0.2M H₂SO₄ is added to each well. Examples of suitable enzyme substrates for use in development of the detection compound include nitrophenylphosphate for alkaline phosphatase or tetramethylbenzidine sulfonate (TMBS) for horseradish peroxidase. The degree of color development, read as Absorbance Units (AU, read at 450 nm in the case of TNBS, or at 405 nm in the case of p-nitrophenol) is indicative of the amount of progastrin present in the test sample, and the actual concentration can be determined by reading absorbance of the test sample against a standard curve generated with known concentrations of SRS or against a standard curve generated with authentic progastrin.

7. Reading: When sufficient assay signal was attained the signal was measured using a microplate reader/spectrophotometer.

8. Data Processing: The assay signals obtained with known standard solutions of the SRS (or progastrin) form were used to construct a calibration curve (signal vs. concentration). The calibration curve was used to interpolate concentrations of the gastrin hormone form in test samples.

EXAMPLE 9. Immunoenzymometric Assay Designed to Measure Progastrin 1-80

The results of the assay for progastrin 1-80 are shown in Table 4. The general methods for this assay are described in Example 8. In this test, wells were coated with affinity purified Mab 490-1, specific for the N terminal end of progastrin. The detection conjugate used was Mab 495-1-HRP conjugate. As the data show, SRS 1 was detected at concentrations down to 100 pM; whereas the closely related SRS 2 was detected only to 100 nM. Therefore, the working range of this assay for progastrin 1-80 would be below 100 nM, and above 10 pM. The negative control peptide Gastrin 17 was not detected. This example demonstrates that the progastrin Mab can be used to assay for progastrin 1-80. This example also demonstrates the utility of SRS 1 as a standard molecule for measuring progastrin 1-80 by ELISA.

TABLE 4: Assay for progastrin 1-80.

Peptide Concentration	Absorbance Values (background subtracted)		
	SRS 1	SRS 2	Gastrin 17
10 µM	NT	0.942	NT
1 µM	NT	0.955	NT
100 nM	0.951	0.109	0.001
10 nM	1.079	0	0.001
1 nM	0.923	0	0.002
100 pM	0.094	0.002	0
10 pM	0.005	0.002	0
1 pM	0	NT	0.001
100 fM	0	NT	0.015

EXAMPLE 10. Immunoenzymometric Assay Designed to Measure Progastrin 6-80

The results of the assay for progastrin 6-80 are shown in Table 5. The general methods for this assay are described in Example 8. In this test, wells were coated with affinity purified Mab 491-1, specific for the N terminal region of progastrin (amino acids 6-14). The detection conjugate used was Mab 495-1-HRP conjugate. As the data show, SRS 2 was detected at concentrations down to

100 pM (as the absorbance at this concentration was above baseline, the assay could detect less than 100 pM); whereas the closely related SRS 1 was detected only to 100 µM. Therefore, the working range of this assay for progastrin 6-80 would be below 100 µM, and above 10 pM. The negative control peptide Gastrin 17 was not detected. This example demonstrates that the progastrin Mab can be used to assay for progastrin 6-80. This example also demonstrates the utility of SRS 2 for measuring progastrin 6-80.

TABLE 5: Assay for progastrin 6-80.

Peptide Concentration	Absorbance Values (background subtracted)		
	SRS 1	SRS 2	Gastrin 17
100 µM	0.191	3.407	0.002
10 µM	0.015	2.324	0.002
1 µM	0.010	2.721	0.002
100 nM	0.010	1.856	0.002
10 nM	0.008	1.431	0.001
1 nM	0.009	0.462	0.013
100 pM	0.004	0.053	0
10 pM	0	NT	0.001
1 pM	0	NT	0.015

NT: Not tested.

EXAMPLE 11. Selection of Optimal Mab for Specific Applications

Despite sharing specificity for a given epitope, different Mabs against a given epitope may differ in their performance in specific applications. Thus, the Mabs must be compared for their activity in each circumstance, in order that the MAb with optimal performance be selected for use in a particular application. This example demonstrates how MAbs specific for the C terminal end of progastrin differ in their ability to serve as progastrin detection antibodies in the immunoenzymometric assay for progastrin and provide an optimal formulation.

The detection antibody reagent in the quantitative assay for progastrin (described in Example 8) is a Mab directed against the C terminal end epitope of progastrin linked to HRP. Four MAbs specific for the C terminal end epitope of progastrin were isolated by cloning from fusion number 495, described above. These were MAbs 495-1, 495-2, 495-3 and 495-4. To prepare HRP conjugates of these MAbs, we followed methodologies familiar to those skilled in the art, by employing our unique components in conjunction with commercially available kits at key steps. Thus, each of MAb 495-1 through -4 were separately produced as ascites MAbs in mice (as described in Mishell

and Shiigi, chapter 17). The presence of a MAb in the ascites fluid was confirmed by direct binding ELISA against the target antigen hProGastrin(72-80)-PPC-BSA, as described above.

MAbs were affinity purified from the ascites fluid by column chromatography over hProGastrin(72-80)-PPC linked to sulfolink gel (Pierce) and eluted with glycine buffer, following the directions supplied with the Pierce Sulfolink Kit. Mabs were further purified by diafiltration in an Amicon filtration unit, and the final protein concentration was determined by A₂₈₀ measurement. Each of the four purified MAb preparations was conjugated to HRP using the Pierce EZ-Link™ Plus kit. After purification, the HRP and antigen binding activity of each Mab-HRP conjugate was checked by direct binding ELISA against the hProGastrin(72-80)-PPC-BSA target antigen, and all were found to be active. Final concentrations of the four conjugates were set at 0.63 to 0.68 mg/mL.

To compare the performance of the four MAb-HRP conjugates in the immunoenzymometric assay for progastrin, the assay was run as described in Example 9 using SRS 1 and in Example 10 using SRS 2. Each of the 495 MAb-HRP conjugates were tested separately in each of the individual assays, as detection reagents for captured SRS 1 and 2. The MAb-HRP conjugates were used at 1:2000 dilutions from the stock solutions. In the assay to detect SRS 2 (for progastrin 6-80), wherein the plate was coated with MAb 491-1 and SRS 2 was thereby captured, the optimal conjugate for detection of the captured SRS 2 was 495-1-HRP. This can be seen in Table 6, wherein 458-1-HRP detected SRS 2 at a concentration of 100 pM. The other three conjugates were less effective, detecting SRS 2 to 1 nM. Thus, the 495-1-HRP conjugate would be used as the detection reagent in the immunoenzymometric assay for progastrin 6-80, using SRS 2, as run under the conditions of this example.

TABLE 6: Comparison of MAb 495-HRP conjugates as Detection Reagents in the Immunoenzymometric assay for Progastrin 6-80

SRS 2 Peptide Concentration	Absorbance Values (background subtracted)			
	495-1- HRP	495-2- HRP	495-3- HRP	495-4-HRP
100 μM	3.407	0.788	3.220	0.809
10 μM	2.324	0.855	1.437	0.671
1 μM	2.721	0.977	1.117	0.582
100 nM	1.856	0.814	0.857	0.571
10 nM	1.431	0.359	0.470	0.254
1 nM	0.462	0.012	0.096	0.029
100 pM	0.053	0	0.007	0.003

Similar results were obtained when the four 495-HRP MAb conjugates were tested in the SRS 1 assay for progastrin 1-80, demonstrating the superiority of the 495-1-HRP conjugate in these assays.

DEPOSIT OF HYBRIDOMA CELL LINES

The following hybridomas were deposited with the American Type Culture Collection (ATCC, Manassas, VA) on September 1, 2004:

1. Hybridoma 490-1 producing MAb 490-1 was assigned accession number PTA-6189.
2. Hybridoma 491-1 producing MAb 491-1 was assigned accession number PTA-6190.
3. Hybridoma 495-1 producing MAb 495-1 was assigned accession number PTA-6191.

INCORPORATION BY REFERENCE

The specifications of the U.S. patents and the texts of each of the references cited herein are hereby incorporated by reference in their entireties.

What is claimed is:

1. A progastrin-binding molecule that selectively binds progastrin, wherein the molecule does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly).
2. The progastrin-binding molecule according to claim 1, wherein the molecule comprises an antibody binding region.
3. The progastrin-binding molecule according to any one of claims 1 or 2, wherein the molecule is an antibody.
4. The progastrin-binding molecule according to any one of claims 1 to 3, wherein the molecule is a monoclonal antibody.
5. The progastrin-binding molecule according to any one of claims 1 to 3, wherein the molecule is a single chain antibody.
6. The progastrin-binding molecule according to any one of claims 1 to 5, wherein the antibody is a mammalian antibody.
7. The progastrin-binding molecule according to any one of claims 1 to 6, wherein the mammalian antibody is a murine antibody.
8. The progastrin-binding molecule according to any one of claims 1 to 6, wherein the mammalian antibody is a chimeric human/mouse antibody, a human antibody or a humanized antibody.
9. A monoclonal antibody that selectively binds an epitope within the amino acid sequence SWKPRSQQP (SEQ ID NO: 6).
10. The monoclonal antibody of claim 9, wherein the antibody binds the epitope bound by the monoclonal antibody produced by the hybridoma 490-1.
11. A monoclonal antibody that selectively binds an epitope within the amino acid sequence SQQPDAPLG (SEQ ID NO: 7).
12. The monoclonal antibody of claim 11, wherein the antibody binds the epitope bound by the monoclonal antibody produced by the hybridoma 491-1.
13. A monoclonal antibody that selectively binds an epitope within the amino acid sequence GRRSAEDEN (SEQ ID NO: 8).
14. The monoclonal antibody of claim 13, wherein the antibody binds the epitope bound by the monoclonal antibody produced by the hybridoma 495-1.

15. A panel of monoclonal antibodies, comprising two or more antibodies selected from the group consisting of an antibody that selectively binds the N-terminus of progastrin at an epitope within the amino acid sequence of SEQ ID NO: 6, an antibody that selectively binds the N-terminus of progastrin at an epitope within the amino acid sequence of SEQ ID NO: 7 and an antibody that selectively binds the C-terminus of progastrin at an epitope within the amino acid sequence of SEQ ID NO: 8.

16. The hybridoma 490-1 (ATCC Accession No. PTA-6189).

17. The hybridoma 491-1 (ATCC Accession No. PTA-6190).

18. The hybridoma 495-1 (ATCC Accession No. PTA-6191).

19. A pharmaceutical composition comprising a monoclonal antibody of any one of claims 1 to 14, and a pharmaceutically acceptable carrier.

20. A pharmaceutical composition of claim 19, wherein the monoclonal antibody is a chimeric human/mouse antibody, a human antibody or a humanized antibody.

21. A progastrin immunoassay, comprising

- a) obtaining a sample to be assayed for progastrin;
- b) contacting the sample with a monoclonal antibody that specifically binds progastrin, but does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly), under suitable conditions for antibody binding and allowing any progastrin present to form a progastrin-monoclonal antibody complex; and
- c) detecting the presence or absence of the progastrin-monoclonal antibody complex; and/or determining the amount of progastrin-monoclonal antibody complex in the sample by an immunodetection method.

22. The progastrin immunoassay according to claim 21, wherein the monoclonal antibody selectively binds an epitope within the amino acid sequence SWKPRSQQP (SEQ ID NO: 6), an epitope within the amino acid sequence SQQPDAPLG (SEQ ID NO: 7), or an epitope within the amino acid sequence GRRSAEDEN (SEQ ID NO: 8).

23. The progastrin immunoassay according to any one of claims 21 or 22, wherein the monoclonal antibody binds the epitope bound by the monoclonal antibody produced by a hybridoma selected from the group consisting of 490-1, 491-1 and 495-1.

24. The progastrin immunoassay according to any one of claims 21 to 23, wherein the immunodetection method is an enzyme-linked immunosorption assay (ELISA), a radioimmunoassay (RIA), an immunohistochemistry (IHC) assay, an immunofluorescence (IF)

assay, an agglutination assay, a western blot assay, a dot blot assay, a slot blot assay or a surface plasmon resistance detection method.

25. A method of diagnosing a gastrin-promoted disease or condition in a patient, comprising determining the level of progastrin in a sample of a biological fluid from the patient and comparing the level of progastrin in the sample with the level of progastrin in a sample of biological fluid from one or more control individuals, or with a reference standard.

26. A method of prevention or treatment of a gastrin-promoted disease or condition comprising administering the pharmaceutical composition of claim 19 to a patient in need thereof.

27. A method of monitoring a gastrin-promoted disease or condition in a patient, comprising determining the level of progastrin in a sample of a biological fluid from a patient suffering from or at risk of a gastrin-promoted disease or condition at a first time point; determining the level of progastrin in one or more samples of the biological fluid from the patient at different time points; and thereby monitoring the gastrin-promoted disease or condition.

28. A kit for performing an immunoassay comprising a monoclonal antibody that selectively binds progastrin, wherein the molecule does not bind gastrin-17(G17), gastrin-34(G34), glycine-extended gastrin-17(G17-Gly), or glycine-extended gastrin-34(G34-Gly) and a suitable container.

29. A surrogate reference standard (SRS) molecule consisting essentially of a peptide chain of from about 10 to about 35 amino acids, wherein the SRS molecule comprises immunomimic of each of at least two epitopes present in a protein of interest of greater than about 50 amino acids.

30. The surrogate reference standard (SRS) molecule according to claim 29, wherein the protein of interest is a peptide hormone or a peptide hormone precursor.

31. The surrogate reference standard (SRS) molecule according to any one of claims 29 or 30, which is a synthetic peptide.

32. The surrogate reference standard (SRS) molecule according to any one of claims 29 to 31, which is a recombinant peptide.

33. The surrogate reference standard (SRS) molecule according to any one of claims 29 to 32, wherein the peptide hormone precursor is progastrin.

34. A method of standardizing a sandwich immunoassay for an protein of interest of greater than about 50 amino acids comprising a first and a second epitope, the method comprising detecting or measuring a signal generated in the immunoassay with a standard amount of a surrogate reference standard (SRS) molecule consisting essentially of a peptide chain of from about 10 to about 35 amino acids comprising immunomimics of the first and second epitopes of the protein of interest.

35. The method according to claim 34, wherein the signal generated in the immunoassay with a standard amount of a surrogate reference standard (SRS) molecule is compared with the signal generated in the immunoassay with a sample comprising an unknown amount of the protein of interest.

36. The method according to any one of claims 34 or 35, wherein the signal generated in the immunoassay with a standard amount of a surrogate reference standard (SRS) molecule is compared with the signal generated in the immunoassay with a sample comprising a known amount of the protein of interest.

37. The method according to any one of claims 34 to 36, wherein the immunoassay is an enzyme-linked immunosorption assay (ELISA), a radioimmunoassay (RIA), an immunohistochemistry (IHC) assay, an immunofluorescence (IF) assay, an agglutination assay, a western blot assay, a dot blot assay, a slot blot assay or a surface plasmon resistance detection method.

38. The method according to any one of claims 34 to 37, wherein the protein of interest is a peptide hormone or a peptide hormone precursor.

39. The method according to any one of claims 34 to 38, wherein the peptide hormone precursor is progastrin.

40. The method according to any one of claims 34 to 39, wherein the SRS molecule is selected from the group consisting of [progastrin 1-9]-[progastrin 72-80] (SWKPRSQQPGRRSAEDEN, SEQ ID NO: 10); [progastrin 6-14]-[progastrin 72-80] (SQQPDAPLGGRRSAEDEN, SEQ ID NO: 11); [progastrin 1-9]-Gly-Gly-[progastrin 72-80] (SWKPRSQQPGGGRRSAEDEN, SEQ ID NO: 12); [progastrin 1-9]-Pro-[progastrin 72-80] (SWKPRSQQPPGRRSAEDEN, SEQ ID NO: 13); [progastrin 1-9]-Pro-Gly-Gly-Pro-Pro-[progastrin 72-80] (SWKPRSQQPPGGPPGRRSAEDEN, SEQ ID NO: 14); and [progastrin 6-14]-Pro-Pro-Gly-Pro-Pro-[progastrin 72-80] (SQQPDAPLGPPGGPPGRRSAEDEN, SEQ ID NO: 15).

Aphoton 0083.WorkFile.txt

Organization Applicant

Street : 1628 JFK Boulevard
 City : Philadelphia
 State :
 Country : Philadelphia, USA
 PostalCode : PA 19103
 PhoneNumber :
 FaxNumber :
 EmailAddress :
<110> OrganizationName : Aphoton Corporation

Application Project

<120> Title : Monoclonal antibodies to progastrin
<130> AppFileReference : Aphoton 0083
<140> CurrentAppNumber :
<141> CurrentFilingDate : _____

Sequence

<213> OrganismName : human
<400> PreSequenceString :
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GPQGPPPHLVA DPSKKQGPWL EEEEEAYGWM DFGRRSAEDE N 101
<212> Type : PRT
<211> Length : 101
SequenceName : Human preprogastrin (SEQ ID No 1)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
EGPWLEEEEEE AYGWMDF 17
<212> Type : PRT
<211> Length : 17
SequenceName : Human mature gastrin G17 (SEQ ID No 2)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
EGPWLEEEEEE AYGWMDFG 18
<212> Type : PRT
<211> Length : 18
SequenceName : Human G17-gly (SEQ ID No 3)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
ELGPQGPPHL VADPSKKEGP WLEEEEEAYG WMDF 34
<212> Type : PRT
<211> Length : 34
SequenceName : Human gastrin G-34 (SEQ ID No 4)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
ELGPQGPPHL VADPSKKEGP WLEEEEEAYG WMDFG 35
<212> Type : PRT
<211> Length : 35
SequenceName : Human gastrin G34-gly (SEQ ID No 5)

Aphton 0083.workfile.txt

SequenceDescription :

Sequence

```
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<213> OrganismName : Human
<400> PreSequenceString :
SWKPRRSQQP
<212> Type : PRT
<211> Length : 9
    SequenceName : progastrin (SEQ ID No 6)
    SequenceDescription :
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9

Sequence

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<213> OrganismName : Human
<400> PreSequenceString :
SQQPDAPLG
<212> Type : PRT
<211> Length : 9
    SequenceName : progastrin (SEQ ID No. 7)
    SequenceDescription :
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9

Sequence

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<213> OrganismName : Human
<400> PreSequenceString :
GRRSAEDEN
<212> Type : PRT
<211> Length : 9
    SequenceName : progastrin (SEQ ID No 8)
    SequenceDescription :
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9

Sequence

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<213> OrganismName : Completely Artificial
<400> PreSequenceString :
PPGGPP
<212> Type : PRT
<211> Length : 6
    SequenceName : linker peptide (SEQ ID No 9)
    SequenceDescription :
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6

Feature

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Sequence: linker peptide (SEQ ID No 9):
<221> FeatureKey : PEPTIDE
<222> LocationFrom : 1
<222> LocationTo : 6
    Other Information :
    CDSJoin : No
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Sequence

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<213> OrganismName : Human
<400> PreSequenceString :
SWKPRRSQQPG RRSaedEN
<212> Type : PRT
<211> Length : 18
    SequenceName : progastrin (SEQ ID No 10)
    SequenceDescription :
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18

Sequence

```
-----
<213> OrganismName : Human
<400> PreSequenceString :
SQQPDAPLGG RRSaedEN
<212> Type : PRT
<211> Length : 18
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18

Aphoton 0083.WorkFile.txt

SequenceName : progastrin (SEQ ID No 11)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SWKPRSQQPG GGRRSAEDEN
<212> Type : PRT
<211> Length : 20
SequenceName : progastrin (SEQ ID No 12)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SWKPRSQQPP GRRSAEDEN
<212> Type : PRT
<211> Length : 19
SequenceName : progastrin (SEQ ID No 13)
SequenceDescription :

Sequence

<213> OrganismName : Human
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SWKPRSQQPP GGPPGRRSAE DEN
<212> Type : PRT
<211> Length : 23
SequenceName : progastrin (SEQ ID No 14)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SQQPDAPLGP PGGPPGRRSA EDEN
<212> Type : PRT
<211> Length : 24
SequenceName : progastrin (SEQ ID No 15)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SWKPRSQQPP C
<212> Type : PRT
<211> Length : 11
SequenceName : progastrin (SEQ ID No 16)
SequenceDescription :

Sequence

<213> OrganismName : Human
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SWKPRSQQP
<212> Type : PRT
<211> Length : 9
SequenceName : progastrin (SEQ ID No 17)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SQQPDAPLGP PC

20

19

23

24

11

9

12

Aphoton 0083.workFile.txt

<212> Type : PRT
<211> Length : 12
SequenceName : progastrin (SEQ ID No 18)
SequenceDescription :

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
SQQPDAPLG
<212> Type : PRT
<211> Length : 9
SequenceName : progastrin (SEQ ID No 19)
SequenceDescription :

9

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
CPPGRRSAED EN
<212> Type : PRT
<211> Length : 12
SequenceName : progastrin (SEQ ID No 20)
SequenceDescription :

12

Sequence

<213> OrganismName : Human
<400> PreSequenceString :
GRRSAEDEN
<212> Type : PRT
<211> Length : 9
SequenceName : progastrin (SEQ ID No 21)
SequenceDescription :

9

INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB2005/002793

A. CLASSIFICATION OF SUBJECT MATTER
 G01N33/53 A61K39/395
 //C07K16/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, EMBASE, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SOBHANI I ET AL: "IMMUNOHISTOCHEMICAL CHARACTERIZATION OF GASTRINOMAS WITH ANTIBODIES SPECIFIC TO DIFFERENT FRAGMENTS OF PROGASTRIN" GASTROENTEROLOGIE CLINIQUE ET BIOLOGIQUE, vol. 13, no. 11, 1989, pages 865-872, XP009057920 ISSN: 0399-8320 page 866	1-28
Y	-----	33, 39, 40
X	VARRO A ET AL: "Pathways of Processing of the Gastrin Precursor in Rat Antral Mucosa" JOURNAL OF CLINICAL INVESTIGATION, vol. 95, no. 4, 1995, pages 1642-1649, XP009057912 ISSN: 0021-9738 page 1643	1-28
Y	-----	33, 39, 40
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

° Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 November 2005	07/12/2005
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Wagner, R

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2005/002793

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/13114 A (BIO-RAD PASTEUR; GARRIC, ISABELLE, NATHALIE; GIULIANI, ISABELLE, KARIN) 22 February 2001 (2001-02-22) page 23 – page 24; claim 25 -----	29-32, 34-38
Y	-----	33,39,40
E	WO 2005/095459 A (APHTON CORP 'US!; GRIMES STEPHEN 'US!; MAKISHIMA RONALD 'US!) 13 October 2005 (2005-10-13) paragraph '0045! -----	1-8
A	SINGH POMILA ET AL: "Incomplete processing of progastrin expressed by human colon cancer cells: Role of noncarboxyamidated gastrins" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 266, no. 3 PART 1, 1994, pages G459-G468, XP009057913 ISSN: 0002-9513 the whole document -----	1-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2005/002793

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 26 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB2005/002793

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO 0113114	A	22-02-2001	CA 2382035 A1 EP 1204865 A1 FR 2797689 A1 JP 2003507711 T	22-02-2001 15-05-2002 23-02-2001 25-02-2003
WO 2005095459	A	13-10-2005	NONE	